

# Ionic effect investigation of a potentiometric sensor for urea and surface morphology observation of entrapped urease/ polypyrrole matrix

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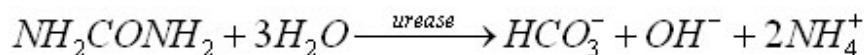
## Abstract

Potential-dynamic polymerization of buffered urease and pyrrole monomer onto carbon papers was conducted to fabricate an immobilized urease electrode for measuring the urea concentration. The potentiometric response corresponding to ammonia, the product formed from the urease catalyzed urea reaction, was employed for the urea concentration measurement. Scanning electron microscopic photographs showed that the polypyrrole matrix appeared to be of in the shape of cylindrical nanotubes. The composite electrodes had high sensitivity in urea detection, showing a response linear to the logarithm of the urea concentration in the range of  $10^{-3}$  to 10 mM. Ionic effect on the sensing of urea solution was investigated. The urease/ polypyrrole/ carbon paper electrode developed in this work showed superior long-term stability and reusability. The detection of urea in serum was also well performed.



## 1. Introduction

Urea concentration in blood is measured as blood urea nitrogen (BUN). It is regarded as a reliable index of kidney diseases. The BUN value for an adult male is 8–21 mg/dL while it is 6–20 mg/dL for an adult female. To measure urea concentration by immobilizing urease onto an electrode is considered to be the most promising approach. The reaction is known as



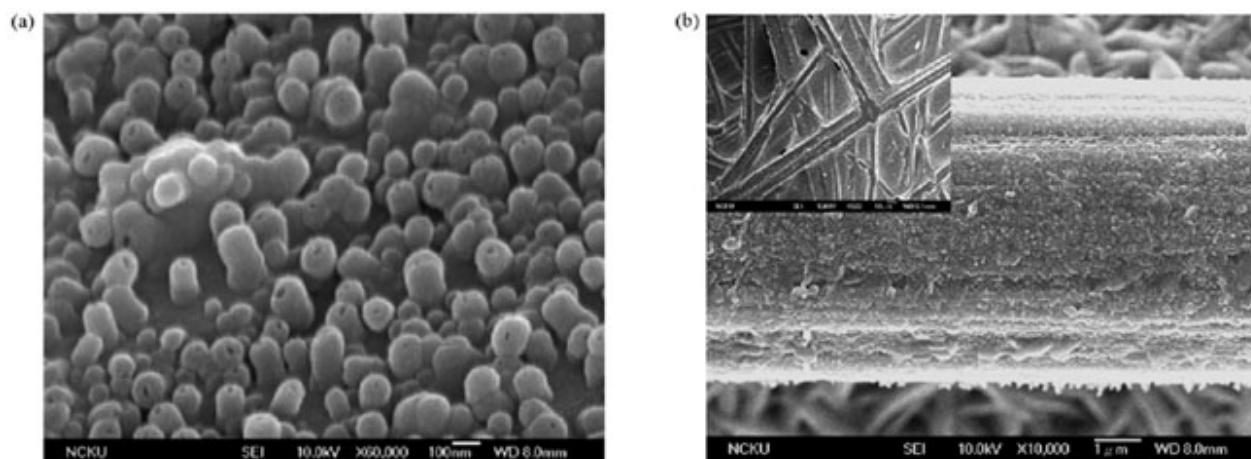
Therefore the formation of ammonia or the change of pH could be measured in the potentiometric mode. Immobilization of enzyme by entrapment or covalent binding within the electro-conducting polymer film was widely investigated in literature. Cosnier reviewed thoroughly the related works regarding this issue. Polypyrrole, polyaniline, poly(*o*-phenylenediamine), polyacetylene, polythiophene, and polyphenol related polymers were all effective conducting polymers for the immobilization of enzymes. The immobilized urease electrode for the potentiometric detection of urea concentration by the formation of ammonium ions was first proposed. Afterwards, there was research work focused on the immobilization of urease onto the electrode for the sensing of urea. Later, using electropolymerization method to fabricate a polymer matrix onto the electrode for the entrapment of urease further gained a lot of attentions. Flow injection analysis (FIA) was further introduced to the urea sensor with urease entrapped in polypyrrole film. Based on polypyrrole-urease film, amperometric detection was carried out. Later, the sensor was further modified and the pulsed-amperometric method was used to detect the blood samples. In 1998, [Walczerz et al.](#) emphasized on using the FIA system for the determination of urea concentration in human serum samples and the results were promising for the synthesis of the pH-membrane electrode. Then, urea concentration of several millimolar could be analyzed with a double-channel FIA system.

In recent years, optical sensor was also proposed for urea sensing. Martelet and Jaffrezic-Renault's team investigated the urea sensor based on ISFETs (ion selective field effect transistors). Rajesh et al. synthesized polypyrrole-based copolymer, poly(N-3-aminopropyl pyrrole-copolyrrole) with urease for the amperometric detection of urea. The response time was very short and the electrode stability could last for 2 months. Other than the conducting polymer materials, the film for the immobilization of urease could also be polyethylenimine and chitosan. Besides of the conducting polymer matrix, inorganic oxide matrix was used to entrap the urease. Additionally, Sahney et al. applied sol-gel such as TMOS for the immobilization of urease to detect urea from the blood samples. The microfluid system was further investigated for the multi-detection of ammonia, creatinine and urea. In recent years, nanotechnology was also incorporated with these biosensors. Polypyrrole film was fabricated onto the TiO<sub>2</sub> electrode first, it then was followed by the modification of polypyrrole film with bovine serum albumin (BSA). The formation of peptide bonds was developed between BSA and urease, thus, the covalent bond could make the urease firmly immobilized onto the electrode. Very recently, Si/SiO<sub>2</sub>/Si<sub>3</sub>N<sub>4</sub> electrode was designed for the Chemical field effect capacitance (ChemFEC) device. Polypyrrole film was then coated onto the electrode by the avidin-biotin affinity. Afterwards, the urease was immobilized onto the film via the ligation with subiotin. Hence, the sensing of urea could be performed from the capacity-potential measurement. There are researches emphasized on different aspects. In this work, the charge density was considered to be an important factor for the electropolymerization. Polypyrrole was used as the matrix to entrap urease because of its superior electro-conducting property. Carbon paper was used because of its fibrous structure for better immobilization and fixation of polymer matrix as well as urease enzyme. Performance of the immobilized urease electrode was also evaluated from the aspects such as sensitivity, stability, and reusability.

## 2. Results and discussion

### 2.1. SEM observation of the surface morphology

Fig. 1(a) is the SEM photograph in 60,000 folds for the morphology of the carbon paper covered with polypyrrole tubes and Fig. 1(b) is the one in 10,000 folds for the surface condition of urease/polypyrrole/carbon paper. The smaller photo in Fig. 1(b) for comparison is the surface morphology of the carbon paper in 500 folds but in a larger area distributed with fibrous matrix. Further immobilization of urease into the polymer matrix, the short polypyrrole tubes (Fig. 1(a) and (b) of Supplementary data) are no longer observed. It is believed that the difference in morphology with and without urease is not really due to the embedding of enzyme but rather the coverage of the thicker polymer film. In our work, the uniformly oriented polypyrrole cylindrical tubes were inspected (Fig. 1(a)) and such morphology was never observed from the literature before. The uniform shape of the grown polypyrrole could be correlated to the good performance of the immobilized urease electrode prepared in this work. (Other photos such as the surfaces of the carbon paper and polypyrrole coated carbon paper enlarged 10,000 folds are provided as Fig. 1(a) and (b) of Supplementary data.)



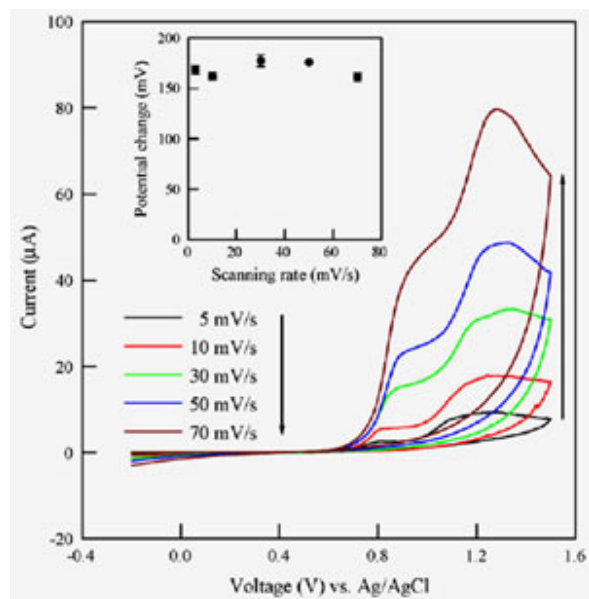
**Fig. 1.** SEM photographs of the surface morphology of (a) polypyrrole nanotube/ carbon paper ( $\times 60,000$ ); (b) urease/polypyrrole/carbon paper ( $\times 10,000$ ).

## 2.2. Effect of scanning rate on the electropolymerization of the immobilized layer for the potentiometric response

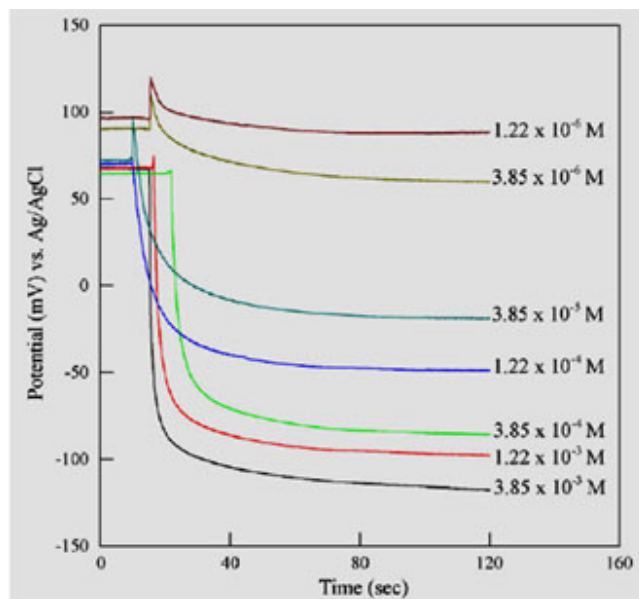
Cyclic voltammetric electropolymerization was applied to fabricate the immobilized urease polypyrrole film onto the carbon paper working electrode. The charge density through the surface of the urease/polypyrrole/carbon paper (CP) working electrode was controlled approximately at  $8 \text{ mC/cm}^2$ . The cyclic voltammograms obtained from these scanning rates are shown in Fig. 2. Largest response current in the profile occurred at a scanning rate of  $70 \text{ mV/s}$ . This could be due to the charging rate of the CP working electrode during the preparation of urease/polypyrrole layer. To operate under a faster scanning rate corresponded to a faster charging rate. Therefore, it would cause the positive charges escape from the surface of the electrode much easily. As a result, the pyrrole monomer would receive the positive charges for oxidation much readily. Therefore, the peak of the oxidation current during the electropolymerization became larger. Significant oxidation peak was observed at a high potential of around  $1.3 \text{ V}$ . To operate with a lower scanning rate, the peak moved away from  $1.3 \text{ V}$  and towards a lower potential. The peak occurred around  $1.3 \text{ V}$  was believed to be caused by the oxidation of pyrrole. The electrodes thus prepared were further used to measure the urea concentration of  $1.0 \text{ mM}$ . The results are plotted in Fig. 2. Since the charge density for the electropolymerization was intentionally controlled the same, the effect from different scanning rates but with the same charge density should be almost the same and the measured data also appeared this way.

## 2.3. Sensing performance evaluation of the urease/polypyrrole/CP electrode

The immobilized urease sensing electrode for the detection of urea concentration was carried out by the electropolymerization of pyrrole monomer and urease in the scanning range of  $-0.2$  to  $1.5 \text{ V}$  with a rate of  $50 \text{ mV/s}$ . Urea concentrations of  $10^{-1}$  to  $10^{-4.5} \text{ M}$  were injected into the working cell. The injected urea caused a corresponding concentration of  $3.85 \text{ mM} - 1.22 \mu \text{M}$  in the solution. Once the urea solution was injected into the system, the open circuit potential dropped down rapidly until a kinetic steady response was reached. Potential change versus urea concentration is plotted in Fig. 3. The response was significant and rapid. Excellent calibration curve of potential change against urea concentration with a sensitivity of  $53.74 \text{ mV/decade}^{-1}$  (with an R.S.D. of 1.71) of very high precision (shown in Supplementary data Fig. 2) was achieved.



**Fig. 2.** Cyclic voltammograms for the electropolymerization of urease entrapped poly-



**Fig. 3.** Time profile of the detected potential change against urea concentration by the immobilized urease/ polypyrrole/

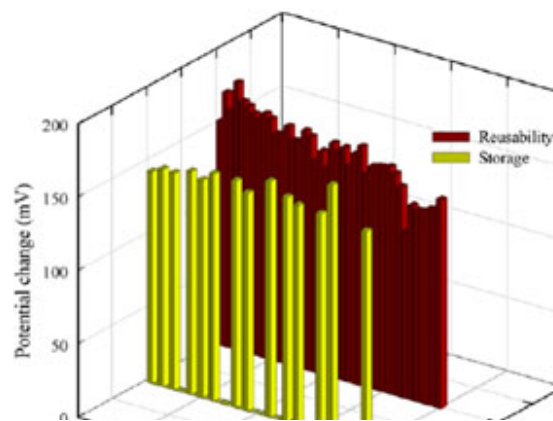
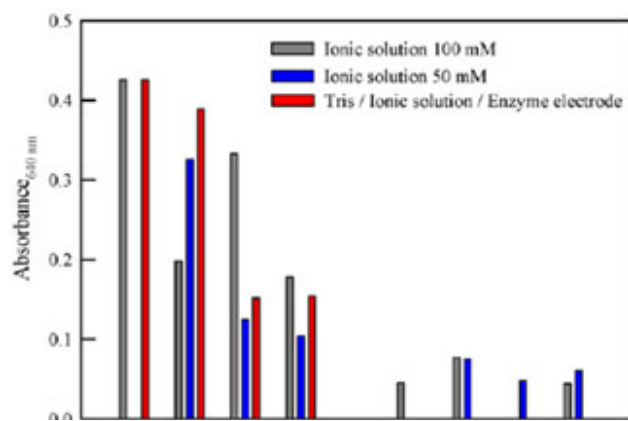
pyrrole onto carbon paper obtained from different scanning rates and the detected potential changes of 1.0 mM urea concentration from the urease immobilized electrodes thus prepared.

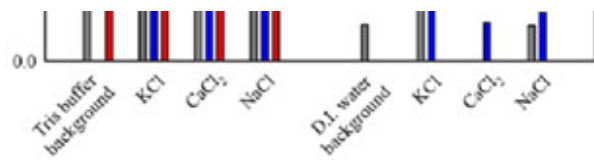
#### 2.4. Ionic effect on the detection of urea by the immobilized urease electrode

The ionic species with high dissociation, such as NaCl, KCl, and CaCl<sub>2</sub>, were used for the investigation of ionic effect on the urea sensing of the prepared electrode. They all have charge numbers of less than or equal to two and therefore could fully dissociate in aqueous solution. Urease contains two nickel (II) covalently bound to the active site of each subunit. It was reported that urease is rather stable because of its zwitter ionic resonance energy and also the stable coordination of the nickel (II) complex. However, the presence of these ions did reduce the amount of potential change of urea by the urease electrode. The concentration of the respective ion was 100 mM. The ions might affect the enzymatic activity of urease as well as the potentiometric signal. To discover the effect, the urease activity from urea substrate in the presence of different ions was also analyzed. The control group for the measurement without urease was also carried out. The results in Fig. 4 indicated that either ion including water may not seem to cause significant contribution to the colorimetric measurement of the urea solution at an applied concentration of 50 mM.

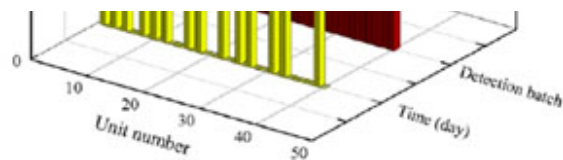
However, added with 100 mM of the ionic solute, precipitate occurred from the one added with CaCl<sub>2</sub>. Thus, there was no data available in the presence of 100 mM CaCl<sub>2</sub>. Obviously, CaCl<sub>2</sub> of 100 mM did cause interference on the colorimetric analysis of the urease activity. Furthermore, to add the ionic species of 100 mM into the buffer solution without the presence of urease, the ions could interfere with the colorimetric measurement of urea by interaction with the coloring reagent. Additionally, as inspected from Fig. 4, all the three ionic species did interfere with the colorimetric measurement of urease activity even without significant observation of precipitates. They could affect the activity of urease or they might simply interfere with the colorimetric measurement. The interference on the colorimetric measurement of the product, indophenol blue, was rather complex and difficult to be specified.

In addition to the effect on the measurement of the enzymatic activity, the ions could also interfere with the potentiometric measurement of urea by the formation of precipitates between the dissociated metal ions and the carbonate (or dissolved CO<sub>2</sub>) produced from the urease catalyzed hydrolysis of urea (shown in Supplementary data Fig. 4). Na<sup>+</sup> has larger charge density and higher activity coefficient (from the extended Debye-Huckel equation) than the other species. Therefore, the presence of NaCl in the urea solution caused a comparably larger reduction on the amount of potential change with respect to the same urea concentration. The influence from CaCl<sub>2</sub> and NaCl did not show significant difference while compared to the difference between KCl and the other species. It was due to the larger ionic strength (0.3 M compared to 0.1 M for the other two species) from CaCl<sub>2</sub> and hence could cause larger ionic effect. However, on the contrary, CaCl<sub>2</sub> has a lower activity coefficient than NaCl. Besides, Ca<sup>2+</sup> has a larger charge number and could be easier to form precipitate during the urease catalyzed hydrolysis of urea. As a result, it appeared a larger interference effect than K<sup>+</sup> (shown in Fig. 4 of Supplementary data).





**Fig. 4.** Colorimetric measurement results of urea with and without urease from the ionic solutions (50 mM and 100 mM, respectively) prepared in water and Tris–HCl buffer, respectively. The ions used for this study were KCl, CaCl<sub>2</sub>, and NaCl. (■ in 100 mM ionic solutions; ■ in 50 mM ionic solutions; ■ in Tris–HCl/ionic solution/urease enzyme).



**Fig. 5.** Long term stability on the storage and the reusability tasks evaluation on the urease immobilized electrode.

### 2.5. Long-term storage stability and reusability task of the electrode

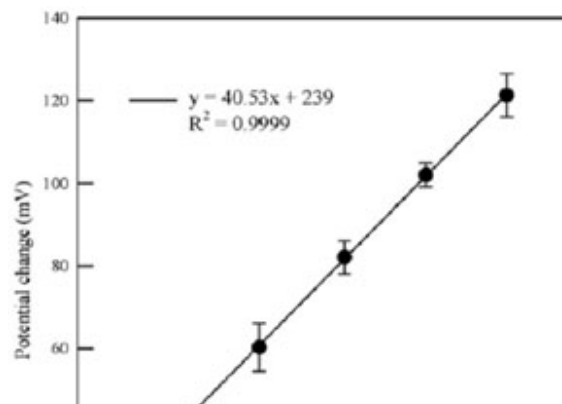
In Fig. 5, the storage stability of the urease/polypyrrole/CP electrode was examined. The potentiometric signals from the immobilized urease electrode were measured with a 1.0 mM urea every 2-3 days. The average relative standard deviation of the data was 4.805. The electrodes were all kept at low temperature. Obviously, nearly the same potential changes with respect to 1.0 mM of urea solutions were measured. The stability test on the immobilized urease electrode was lasted for at least 39 days. It can be inspected from Fig. 5 that the stability of the electrode was still well maintained. (The standard deviations of the data are provided in Table 1 of Supplementary data.) Again in Fig. 5, a single piece of urease electrode was used to detect urea concentration of 1.0 mM. Five minutes were set for each detection cycle. After the detection, the electrode was washed with deionized water. Excellent reusability from the same electrode was performed. The comparison of the urease electrode developed in this work with the other urease electrodes proposed in literature is listed in Table 1. Concluded from the evaluation of the regarding aspects, such as sensitivity, limit of detection (LOD), linear range, stability and reusability, the urease/polypyrrole/carbon paper sensing electrode was already successfully prepared for the determination of urea concentration.

**Table 1** Comparison of the urease electrodes prepared in this work those reported in literature.

Sensing mode	Materials	Sensitivity	LOD	Stability	Response time	Reusability	Dynamic (linear) range	Authors/references
pH potentiometric	Electroinactive polypyrrole/polyurea complex	110 mV/decade <sup>-1</sup>	0.02 mM	-	20 s	90% after 10th use	0.3–30 mM	Osaka et al. (1996)
Potentiometric	Electroinactive polypyrrole	31.8 mV/decade <sup>-1</sup>	-	-	~7 min	-	0.1–30 mM	Kemaba et al. (1997)
Potentiometric, FIA system	Ammonium ion-selective membrane	3.25 mV/mM, 1.53 mV/mM	1 mM	2 months	1.5–3 min	-	0.5–8 mM (single channel); 1–10 mM (double channel)	Walker et al. (1998)
NH <sub>4</sub> potentiometric, FIA system	γ-Alumina/matrix ion-selective membranes	54.2 mV/decade <sup>-1</sup> ~413 mV/mM	0.01 mM 1 mM	3 months 2 months	3 min 6 min	- Over 65% after 1 month	0.03–14 mM 1–13 mM	Liu et al. (1997) Klonck et al. (1999)
NH <sub>4</sub> potentiometric	Chitosan membranes	56 mV/decade <sup>-1</sup>	-	2 months	0.5–2 min	-	0.1–10 mM	Magalhães and Machado (1998)
Potentiometric	Mercaptohydroquinone-modified gold electrode	-	0.2 mM	5 days	8 s	30% after 14 days	~5 mM	Mizutani et al. (1997)
pH potentiometric	Sol-gel film, microencapsulation	11.07 mV/decade <sup>-1</sup>	52 µg/mL	-	5 min	80% after 25 days	0.01–30 mM	Sahney et al. (2006)
Potentiometric	Poly(N-3-aminopropylpyrrole-co-pyrrole)	27.1 mV/decade <sup>-1</sup>	-	2 months	25–50 s	80% after 2 months	6.3 µM–0.407 mM	Rajesh et al. (2005a,b,c)
Potentiometric	PVA/SbQ membrane	-	1 mM	3 months	-	-	1–80 mM	Soldatkin et al. (2003)
NH <sub>4</sub> potentiometric	Polymeric membrane/zeolite	32 mV/pNH <sub>4</sub> <sup>+</sup> , 15 mV/purea	0.03 mM	15 days	5 min	-	0.03–5 mM	Hassanlou et al. (2002)
Potentiometric	Solid-state, PVC-NH <sub>2</sub> membrane	48 ± 5 mV/decade <sup>-1</sup>	0.03 mM	1 month	t <sub>90</sub> < 10 s	-	0.5–50 mM	Taklic et al. (2002)
Potentiometric	Polypyrrole/carbon paper	52.43 mV/decade <sup>-1</sup>	1 µM	>1 month	60–100 s	80% after 40th use	1.22 µM–3.85 mM	This work

### 2.6. Detection of urea in serum sample

The immobilized urease electrode was further used for the detection of urea concentration in the serum samples. Fig. 6 is the calibration curve obtained from the measurement. A sensitivity of 40.53 mV/decade<sup>-1</sup> (with an R.S.D. of 1.363) was resulted from this effort. The detection sensitivity was reduced in serum as expected. The serum contains complex species which could interfere with the detection of urea and



therefore the detection of ammonia ions. Consequently, it could be concluded that the detection of urea in serum samples by the fabricated urease/ polypyrrole/ carbon paper electrode is feasible.

### 3. Conclusions

Polypyrrole film was fabricated onto the carbon paper by cyclic voltammetric electropolymerization for the immobilization of urease. The morphology of uniformly distributed polypyrrole nanotubes onto carbon paper substrate could also be observed from the SEM photographs. Thus, the electrode could be used for the determination of urea concentration. In this work, a detection sensitivity of  $53.74 \text{ mVdecade}^{-1}$  was obtained from the analysis and it was superior to most of the values reported in literature. The limit of detection on the urea concentration was  $1.0 \mu\text{M}$ , which was comparably much lower than the others reported. By controlling at the same charge density during the electro-synthesis of the polymer film, detection of urea concentration performed could achieve almost the same results, which demonstrated an excellent repeatability. In addition, the calibration in the range of  $1.22 \mu\text{M}$ – $3.85 \text{ mM}$  with very high precision was obtained, which was comparably superior to the other reported data. The rapid response time of 60–100 s was achieved in this work. The detection in water and in buffer solution both was proved experimentally to be feasible. The presence of different ions of high dissociation did interfere the electric sensing of urea. Besides, the ions also affected the coloring reaction of the urease catalyzed urea hydrolysis.

The storage stability of this immobilized urease electrode was also well maintained for over a month (39 days) with an average standard deviation of 4.805. The reusability was examined for over 40 detection cycles by the same piece of urease electrode with still 80% of the initial intensity left. The detection of urea concentration in serum samples could still obtain a sensitivity of  $40.53 \text{ mVdecade}^{-1}$ . In summary, the urease electrode for urea concentration was successfully prepared.

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### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.bios.2009.01.036](https://doi.org/10.1016/j.bios.2009.01.036).

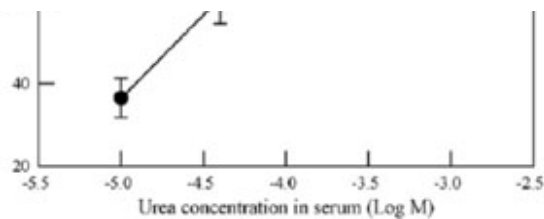


Fig. 6. Detection of urea concentration in serum samples.